(19) [logo] **European Patent Office**

[bar code]
(11) Publication Number: **0 416 572 A1**

(12) **EUROPEAN PATENT APPLICATION**

(21) Application number: **90117061.3**

(22) Date of application: **9/5/90**

(51) Int. Cl.[5]: **C12N 15/82,** C12N 15/11, C07H 21/02, A01H 5/00, A01H 5/10

There is a request to amend the description according to Rule 88, European Patent Convention. A decision on this application will be made in the course of the process before the Examination Department (Guidelines for examination in the EPO, A-V, 2.2).

The applicant has submitted a sequence protocol later and has declared that it contains no new statements.

(30) Priority: **9/7/89 DE 3929741**

(43) Date of publication of the application: **3/13/91 Patent Gazette 91/11**

(84) Specified contracting states: **AT BE CH DE DK ES FR GB IT LI NL SE**

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(54) RNA with endoribonuclease activity against mRNA of maturation genes, its production and its use in plants

(57) Ribozyme genes or gene fragments can be synthesized on the basis of the c-DNA of maturation genes. Then they are inserted into plant cells and expressed there, so that they cause almost complete inhibition of the maturation enzymes.

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RNA WITH ENDORIBONUCLEASE ACTIVITY AGAINST mRNA OF MATURATION GENES, ITS PRODUCTION AND ITS USE IN PLANTS

Under certain conditions, RNA molecules can catalyze reactions of other RNA molecules without involvement of proteins, or autocatalytically split off fragments of their own molecules. For instance, an intron with 413 nucleotides is removed autocatalytically from the 3' end of the 23s rRNA of *Tetrahymena thermophila* and transformed into a circular form. That is accomplished in a series of phosphoester transfer reactions involving guanosine cofactors (Cech, T. R., Nature 30, 578–583 (1983)). Depending on the RNA substrate or the selected reaction conditions, the intron can function as a specific ribonuclease, terminal transferase, phosphotransferase or acid phosphatase. In these processes the RNA molecule can carry out some transformations without being changed itself, acting in that respect like an enzyme. Therefore the concept of ribozyme has been presented for RNA molecules with these properties.

Similar reactions not involving proteins have also been shown for some viroid RNAs and satellite RNAs. For example, self-processing appears to be an essential reaction for multiplication for avocado sunblotch viroid (ASBV) (Hutchins, C. J., et al., Nucleic Acids Res. 14, 3627–3640 (1986)), satellite RNA of tobacco ringspot virus (sTobRv) (Prody, G. A., et al., Science 231, 1577–1580 (1986)) and satellite RNA of lucerne transient streak virus (sLTSV) (Forster, A. C., et al., Cell 49, 211–220 (1987)). Presumably circular forms are produced during the replication of these RNAs, and they serve as "templates" for synthesis of RNAs with excess lengths. These transcripts are tailored to the correct genome length by the autocatalyzed endonucleolytic reactions.

The structures that the RNAs presumably take on for the reaction were described as "hammerheads" (Forster, A. C., et al., Cell 49, 211–220 (1987); Haseloff, J., et al., Nature 334, 585–591 (1988)).

The restriction sites for these RNA enzymes are specific and must have certain structural prerequisites for processing to occur.

It has now been found that ribozymes can attack plant RNA coding for maturation enzymes, and so can be used to influence the maturation process in plants.

Regulation of expression of the DNA coding for the maturation enzyme polygalacturonase by "antisense" RNA was described by Smith, C. J. S., et al., in Nature 334, 724 (1988). A fragment of the polygalacturonase cDNA was included in reverse orientation in an expression vector. Tomato stem segments were transformed by this vector plasmid through *E. coli* and *Agrobacterium tumefaciens*. Then the expression of antisense RNA can be shown in the leaves of the tomato plant. It is assumed that the "antisense" RNA binds to the true polygalacturonase RNA, inactivating it, which it turn results in the polygalacturonase synthesis being partially inhibited.

Now ribozymes are being developed to influence specifically the maturation processes in plants. They bind to the maturation enzyme RNA, and can cut it at certain restriction sites in the sequence. By using the ribozymes according to the invention, the synthesis of certain maturation enzymes can be inhibited not only partially but almost completely, i. e., about 80–100%. Effective inhibitors are described by S. Billich et al., J. Biol. Chem., 34 (1988), 17905–17098 [sic]; M. Moore et al., Biochem. Biophys. Res. Comm., 159, (1989), 420–425; A. D. Richards, R. Roberts, B. M. Dunn, M. C. Graves and J. Kay, FEBS Lett., 247 (1989), 113–117.

High doses of Pepstatin A were able to reduce the biosynthetic formation of the nuclear protein p24 (V. D. Helm, L. Gürtler, J. Eberle and F. Deinhardt, FEBS Lett., 247, (1989), 349–352).

Now a new structural class has been found, which shows highly active inhibition of HIV protease in an enzyme test.

The present invention concerns compounds of Formula I

[See original for structure.]

(I)

in which
Y represents oxygen, sulfur, a group having Formula II or a group of Formula II

[See original for structures.]

(II) (III)

l and m, independently of each other, are 0 or 1;
A is a group having Formula IV and A* is a group having formula IV*.
There are nucleotides which, considered as a whole, are complementary to a DNA sequence of the maturation enzyme to be inhibited and in which the beginning and ending sequences of the oligonucleotide are separated by an intervening RNA sequence consisting in part of specific nucleotides required for the functionality of the ribozyme and in part of variable nucleotides. The ribozyme hybridized with substrate RNA can appear as shown in the following scheme.

[See original for sequence.]

← Substrate RNA

Ribozyme

Loop

in which
N represents nucleotides of the substrate RNA, A, C, G or T
K represents nucleotides complementary to N in the ribozyme
V represents variable nucleotides in the ribozyme, and
V_L represents variable nucleotides in the loop of the ribozyme.
The number of nucleotides V_L in the loop can be from 0 to 550. The GU recognition sequence is preferably selected as the restriction site in the substrate RNA.

The oligonucleotides mentioned are given an appropriate linker. For example, restriction sites of EcoRI, Sall, BamHI, HindIII, EcoRV, Smal, Xhol, and Kpnl have such linkers, preferably Xbal or Pstl.

The oligonucleotides synthesized are cloned by means of the vectors pUC19, pUC 18 or pBluescript (Stratagene, Heidelberg, Product Information), and sequenced.

The verified oligonucleotide is cloned in an intermediary vector with plant promoter. Such vectors include, for instance, the plasmids pPCV701 (Velten, J., et al., EMBO J. 3, 2723–2730 (1984)), pNCN (Fromm, M., et al., PNAS 82, 5824–5826, (1985)) or pNOS (An G. et al., EMBO J. 4, 277–276 (1985)). The preferred vector is pDH51 (Pietrzak, M., et al., Nucleic Acids Res. 14, 5857, (1986)), used with a 35S promoter.

After subsequent transformation of *E. coli*, such as, for instance, *E. coli* MC 1061, DH1, DK1, GM48 or XL-1, positive clones are identified by methods which are themselves known (Maniatis et al., Lab. Manual), such as plasmid minipreparation and restriction with an appropriate restriction enzyme.

Then these positive clones are subcloned in a binary plant vector. pGV3850 (Zambrysk, P., et al., EMBO J. 2, 2143–2150 (1983)) or pOCA18 (Olszewski, N., Nucleic Acids Res. 16, 10785–10782 [sic], (1988)) can be used as plant vectors. It is advantageous to work with pOCA18.

The binary plant vectors obtained, which contain a plant promoter with the attached DNA fragment for ribozyme production in the T-DNA, are used to transform plants. That can be accomplished with techniques such as electroporation or microinjection.

Cocultivation of protoplasts, or transformation of leaf pieces with agrobacteria, are used preferentially. For that purpose, the plant vector construct is transferred by transformation with purified DNA or, by means of a helper strain such as *E. coli* SM10 (Simon, R., et al., Biotechnology 1, 784–791 (1983)), into *Agrobacterium tumefaciens* such as A282 with a Ti plasmid through a "triparental mating." Direct transformation and triparental mating were accomplished as described in "Plant Molecular Biology Manual" (Kluwer Academic Publishers, Dordrecht (1988)).

Essentially all plants can be transformed with binary plant vectors carrying ribozyme-DNA. Dicotyledenous plants are preferred, especially crop plants, such as fruit-bearing plants. Examples include tomato, strawberry, and avocado, as well as plants bearing tropical fruits, such as papaya and mango, as well as pear, apple, nectarine, apricot or peach. The process described is preferably carried out with the tomato. The transformed cells are selected by means of a selection medium, cultured to produce a callus, and regenerated to plants on an appropriate medium (Shain et al., Theor. Appl. Genet. 72, 770–770 [sic] (1986); Masson, J., et al., Plant Science 53, 167–176 (1987), Zhan et al., Plant Mol. Biol. 11, 551–559 (1988); McGranaham et al., Bio/Technology 6, 800–804 (1988), Novrate et al., Bio/Technol. 7, 154–159 (1989)).

The resulting plants are altered by the transformation to the extent that the ribozymes are expressed in the cell. That, in turn, results in the ribozyme RNA binding not only to the RNA complementary to the corresponding maturation genes and causing more or less intense inhibition of the synthesis of the maturation enzymes, but also causes restriction of the RNA complementary to the corresponding maturation genes specifically at GUC sequences, so that synthesis of the maturation genes of concern is almost completely inhibited.

Formation of the ribozyme-specific secondary structural features of the ribozyme-RNA synthesized *in vivo* in the transgenic plants was not at all expectable, so that the observed inhibition of the synthesis of the maturation enzymes was completely surprising.

The following examples provide further explanation of the invention.

Examples

Percentages stated are by weight unless otherwise specified.

1. Cloning the oligonucleotides

The oligonucleotides are synthesized for ribozyme expression on the basis of the following cDNA sequences:

a) 5' TGATGGAGTCCATGTATCA 3'
Segment from the polygalact. [sic] cDNA sequence according to Grierson, D., et al., Nucleic Acids Res. 14, 8595–8603

b) 5' TAGCAAGTCCTGACCTAA 3'
Segment from the cDNA sequence for pectin esterase according to Ray, J., Eur. J. Biochem. 174, 119–124 (1988)

c) 5' TGCTTTGTCCGATACAGT 3'
Sequence segment of the cDNA of a "ripening related protein" according to Ray, J., Nucleic Acids Res. 15, 10587 (1987)

The following nucleotides were synthesized using the phosphoramidite method (Engels, J., et al., Advances in Biochemical Engineering Biotechnology, Vol. 37; Ed.: A. Flechter, Springer Verlag, Berlin/Heidelberg, 1988):

for a): [See original for sequences.]

for b):

for c):

The vector pDH51 (Pietrzak, M., et al., Nucleic Acids Res. 14, 5857 19 [sic]) was cut with the restriction endonucleases Xbal and Pstl, incubated with "calf intestinal phosphatase" (CIP), phenolized and precipitated (Maniatis, Lab. Manual). The vector treated in that manner was ligated with a three-fold excess of phosphorylated oligonucleotides and transformed in *E. coli* MC1061. Positive clones were identified by plasmid minipreparations and subsequent digestion with Xbal and Pstl.

Furthermore, the ampicillin-resistant transformed *E. coli* cells (100 µg ampicillin per ml of LB medium) were transferred to nitrocellulose membranes (Gene Screen Plus®, NEN®, Boston) and incubated on LB medium with ampicillin for a further 14 hours at 37 °C. Then the colonies were disintegrated in 0.5 M NaOH and fixed. After drying, the filters were hybridized with radioactively labeled oligonucleotides. Positive clones produced blackening of the film.

2. Subcloning of a 35S promoter gene fragment in pOCA 18

A 0.75 kb EcoRI fragment was isolated from each of the clones obtained in 1. It was incorporated into a pOCA 18 vector cut with EcoRI and transformed in *E. coli* MC1061. Positive clones were identified by plasmid minipreparations and after subsequent hydrolysis with EcoRI by the appearance of the 0.75 kb bands.

3. Transformation of *Agrobacterium tumefaciens*

The construct obtained in 2. must be transferred into an Agrobacterium to be able to transform plants. That is accomplished either by "triparental mating" or directly. For "triparental mating," 100 µl portions of *E. coli* SM10 from overnight cultures carrying the construct *E. coli* MC1061 and *Agrobacterium tumefaciens* were centrifuged off and suspended together in 30 µl of LB medium. After 30 minutes at room temperature, these bacterial suspensions were applied to a filter on an LB plate without antibiotic. After incubation for 12 hours at 37 °C, the filter was washed with 2.5 ml 10 mM $MgCl_2$. Aliquots were selected on LB plates with rifampicin, tetracycline and kanamycin. Positive colonies were identified by hybridization with ^{32}P-labeled DNA of the gene to be expressed.

For the direct transformation of agrobacteria, the cells were cultured overnight at 28 °C in YEB medium (1% yeast extract, 1% peptone, 0.5% NaCl) with 25 µg/ml kanamycin and 100 µg/ml rifampicin. After 16 hours the bacterial suspension was diluted to an A_{550} of 0.1 and further incubated at 28 °C to an A_{550} of 0.5. 1 ml of this culture was centrifuged off and washed with 1 ml 150 mM NaCl. After the washing, the precipitate was resuspended in 600 µl ice-cold 10 mM $CaCl_2$ solution.

After the cells of the *E. coli* clone obtained in 2. were disintegrated with 0.2 N NaOH/1% SDS, the pOCA 18 vector with the cloned 0.75 kb fragment was isolated and purified by centrifugation in a CsCl density gradient. 1 µg of the plasmid DNA was added to the competent agrobacteria and the

Eppendorf vessel was placed on ice for 1 hour. After 1 hour, the plasmid solution was incubated for 5 minutes at 37 °C and 2 ml YEB medium was added. Then the cells were incubated overnight at 28 °C in a shaking incubator. Then 100 µl portions were placed on YEB plates with 100 µg/ml rifampicin, 25 µg/ml kanamycin and 2.5 µg/ml tetracycline. Colonies appeared on the plates after incubation for 2 days. Positive clones were detected by hybridizing with the corresponding ^{32}P-labeled oligonucleotides a), b) or c).

That was done by plating the transformed agrobacteria onto Gene Screen Plus membranes and incubating them on YEB plates with 100 µg/ml rifampicin, 25 µg/ml kanamycin and 2.5 µg/ml tetracycline for 14 hours at 28 °C. Then the membranes were placed on 0.5 M NaOH for 2 minutes and after that on 0.5 M Tris, pH 7.5, for 2 minutes. After drying, they were prehybridized in 10% dextran sulfate/1 M NaCl/1% SDS for 2 hours at 55 °C together with the radioactively labeled oligonucleotides. After washing at 55 °C for 30 minutes each with 1x SSC (0.15 M NaCl, 0.015 M sodium citrate, pH 7.0) and twice with 0.2-fold SSC, the positive clones were identified by blackening of a film placed on them.

4. Transformation of tomatoes

a) Protoplast transformation:
Tomato protoplasts (Plant Cell Reports 6, 172–175 (1987)) were washed once with W5 solution (154 mM NaCl, 125 mM $CaCl_2 \cdot H_2O$, 5 mM KCl, 5 mM glucose) and once with MaMg solution (0.45 M mannitol, 25 mM $MgCl_2$, 0.1% 2-(N-morpholino)ethanesulfonic acid (MES, pH 5.8), Sigma Chemie, Deisenhofen, Federal Republic of Germany). After careful centrifugation for 3 minutes at 600 rpm, the supernatant was withdrawn except for 0.5 ml. 50 µg calf thymus DNA, 10 µg of the plasmid described, and 10 drops of 45% polyethylene glycol (PEG 8000) were added by pipet. After 10 minutes, the protoplasts were washed twice with W5 solution and incubated in LCM medium (Plant Cell Reports 6, 172–175 (1987)).

b) Leaf fragment transformation with agrobacteria:
Tomato leaves were cut into small pieces which were laid onto MS medium (Murashige, T., et al., Physiol. Plant 15, 473–497 (1962)) with 2% sucrose and 1 ppm of the plant growth hormone Zeatin (Serva Feinbiochemica GmbH & Co., Heidelberg, Federal Republic of Germany) and incubated for a period of 16 hours light/day. They were infected with agrobacteria one day later. The leaf fragments were immersed briefly in a dilute suspension (A 0.15) of the transformed *Agrobacterium* strain, replaced on the same plates, and incubation was continued under the same conditions.
On the third day, all the leaf fragments were washed with a 250 mg/liter solution of carbenicillin and laid on a 2MS medium with 1 ppm Zeatin, 200 mg/liter Cefotaxim (Hoechst AG, Frankfurt), 200 mg/liter carbenicillin and 100 mg/liter kanamycin. After about 20 more days, regenerants were transferred onto 2 MS medium with 1 ppm Zeatin, 200 mg/liter Cefotaxim, 200 mg/liter carbenicillin and 100 mg/ml kanamycin.

Detection of the ribozyme RNA directed against the "ripening related protein" RNA, from a transgenic tomato plant.

All the cellular RNA was isolated from one leaf fragment of a transgenic tomato plant that had been transformed with the gene coding against the "ripening related protein" RNA. That was done by grinding the leaf material in a mortar and pestle under liquid nitrogen. Then, to the powdered leaf material, twice its volume of an extraction buffer (0.2 M sodium acetate, 1% SDS, 10 mM EDTA), twice its volume of phenol (equilibrated with extraction buffer) and half its volume of chloroform/isoamyl alcohol 24:1 (v/v) were added. After very thorough mixing, the phases were separated by centrifuging and the phenolic extraction of the upper aqueous phase was repeated. This aqueous phase was mixed thoroughly and separated by centrifuging again, transferred into a fresh Eppendorf vessel and extracted with chloroform/isoamyl alcohol 24:1 (v/v). Then 1/3 volume of 8 M LiCl was added to the aqueous phase and the solution was held overnight at 4 °C. After centrifuging, a precipitate formed. It was suspended in water and, after adding 0.3 M sodium acetate (pH 5.5), was washed with 2 ½ volumes of ethanol. After washing with 70% ethanol and drying, the precipitate was suspended in 50 µl water.

To demonstrate the specific expression of the gene coding for the ribozyme, about 4 µg each of the total RNA from a non-transformed wild type tomato plant and from a transgenic tomato plant were coated onto a 1% agarose gel with 6% formaldehyde. For the coating, the RNA was dried, suspended in 50% formamide/6% formaldehyde, and heated at 60 °C for 15 minutes.

After the run the agarose gel was washed briefly with water and transferred to a Gene Screen Plus membrane with 10 x SSC. After 24 hours, the membrane was washed with 2 x SSC, incubated for 2 hours at 80 °C, and dried.

After 2 hours prehybridization at 55 °C with 1% SDS, 1 M NaCl and 10% dextran sulfate, hybridization was done with radioactively labeled oligonucleotide c) from the "ripening related protein" DNA.

Demonstration of the gene coding for ribozymes against the "ripening related protein" RNA

Two leaf fragments from the transgenic tomato plant, and from a non-transformed wild type tomato, were ground with a mortar and pestle under liquid nitrogen. The powder was placed in Eppendorf vessels and mixed with 500 µl 2 x CTAB buffer (2 x CTAB: 2% cetyltrimethylammonium bromide, 100 mM Tris, pH 8.0, 20 mM EDTA, 1.4 M NaCl, 1% polyvinylpyrrolidone, molecular weight = 40,000) previously heated to 65 °C. Then 500 µl chloroform/isoamyl alcohol 24:1 (v/v) was added and the aqueous phase was extracted. Then the two phases were separated by centrifuging. The aqueous phase was pipetted into a new Eppendorf vessel and 100 µl 5% CTAB that had been heated to 65 °C was added. Another extraction was done with chloroform/isoamyl alcohol before addition of 500 µl CTAB precipitation buffer (1% CTAB, 50 mM Tris, pH 8.0, 10 mM EDTA) to the aqueous phase. After centrifuging, the precipitate was dissolved in high-salt TE (10 mM Tris pH 8.0, 1 mM EDTA, 1 M NaCl) and precipitated with 2 ½ volumes of ethanol. After centrifuging, washing and drying, the precipitate was suspended in water and treated with 25 µg/ml (final concentration) RNAse A. Then the RNAse was removed by phenolizing. After another drying, the DNA was suspended in 50 µl water.

Then 4 µg of the DNA was hydrolyzed by the restriction endonuclease EcoRI for 1 hour at 37 °C. The hydrolyzate was separated on a 1% agarose gel. The gel was shaken for 30 minutes with 0.4 N NaOH/0.6 M NaCl and then for 30 minutes with 0.5 M Tris Cl [sic] pH 7.5/1.5 M NaCl. A Pstl hydrolyzate of the λ phage DNA was used as the size standard.

Then the DNA was transferred to a Gene Screen Plus membrane with 10 x SSC by means of a capillary blot. Then the filter was dried and prehybridized with 1% SDS/1 M NaCl/10% dextran sulfate. For the hybridization, the prehybrization mixture was mixed with a radioactively labeled sample of oligonucleotide c) that had previously been boiled for 10 minutes.

The ribozyme-coding gene was demonstrated by occurrence of blackening at about 0.8 kb on the X-ray film that had been laid on.

Demonstration of the *in vitro* activity of the ribozyme

The oligonucleotide that coded for a ribozyme against the "ripening related protein" RNA was cloned in the bluescript vector opened up after hydrolysis with the restriction endonucleases Xbal and Pstl. In parallel with that, a DNA fragment of the "ripening related protein" was cloned at the Sacl/Kpnl restriction site in the same vector (Nucleotide numbers 792–815 of the DNA sequence published by Ray, J., et al., Nucleic Acids Res. 15, 10587 (1987)[]). Both vectors were used as DNA matrices for the RNA synthesis in an *in vitro* RNA polymerase reaction.

That was done by cutting the vector carrying the ribozyme gene and the vector carrying the DNA fragment of the "ripening related protein" gene in parallel with the restriction endonuclease Sacl. Then 1 µg portions of the opened vectors were incubated for 30 minutes at 37 °C with 10 uM each of the nucleotides ATP, GTP, CTP, UTP and (α-^{32}P)UTP (5 ...) in 50 mM HEPES (pH 7.5) and 10 µ [sic] T7 RNA polymerase. The RNA was precipitated in ethanol after DNAse treatment.

The synthesized RNA of the ribozyme gene-carrying vector and the RNA of the vector carrying the "ripening related protein" gene fragment were incubated together for two hours at 25 °C in 50 mM Tris•Cl [sic] (pH 7.8) and 10 mM $MgCl_2$. Then the reaction products were separated on a 5% denaturing polyacrylamide gel (8 M urea) and identified by autoradiography on an X-ray film. The autoradiogram showed that the RNA transcript of the "ripening related protein" gene fragment was cut in the presence of ribozyme-RNA.

Demonstration of the delayed maturation of transgenic tomatoes

Comparison of a non-transformed wild type tomato plant with a transgenic tomato plant carrying the gene coding against the "ripening related protein" RNA showed that the tomatoes of the transgenic plant exhibited several days' delay of ripening. The ribozyme activity and the maturing delay linked with it were thus detected also in the tomato fruit, which is the actual site of action.

Claims

1. Ribozyme-coding gene or gene fragment having the DNA sequence

   a) [See original for sequences.]

   b)

   c)

2. RNA with ribozyme activity from the sequence,

   [See original for sequence.]

   Ribozyme

   Loop

   in which K represents nucleotides A, C, G or U complementary to the plant maturation enzyme RNA,
   V represents variable nucleotides A, C, G or U, and
   V_L represents variable nucleotides A, C, G or U in the loop, whereby the number of nucleotides of V_L in the loop is a number from 0 to 550.

3. RNA with ribozyme activity having the sequence

a) [See original for sequences.]

b)

c)

4. Process for producing a ribozyme-coding gene or gene fragment with the DNA sequence

a) [See original for sequences.]

b)

c)

by synthesis of oligonucleotides, characterized in that oligonucleotides each having initial and final sequences of 5, preferably 7 to 10 nucleotides, are synthesized which, considered as a whole, are complementary to a DNA sequence of the maturation enzyme to be inhibited and which are separated by the intervening DNA sequence, consisting in part of specific nucleotides required for the functionality of the ribozyme and in part of variable nucleotides.

5. Process for producing RNA with ribozyme activity from the sequence

[See original for sequence.]

Ribozyme

Loop

characterized in that an oligonucleotide of the sequence

[See original for sequences.]

is synthesized, in which
K represents nucleotides A, C, G or T complementary to plant maturation enzyme RNA,
V represents variable nucleotides A, C, G or T,
V_L represents variable nucleotides A, C, G or T, whereby the nucleotide number of V_L is a number from 0 to 550 and

K', V', V_L' are each nucleotides A, C, G or T complementary to K, V, and V_L,

and is cloned in an intermediate vector with a plant promoter, then cloned together with the plant promoter in a binary plant vector and is transformed with the plasmid DNA of a plant so obtained.

6. Process according to Claim 5, characterized in that an RNA of the sequence

a) [See original for sequence.]

b) [See original for sequences.]

or

c)

is synthesized.

7. Plant cells, plants, their seeds and parts, containing one or more of the DNA sequences according to Claim 1.

8. Plant cells, plants, their seeds and parts, containing one or more of the RNA sequences according one of Claims 2 or 3.

9. Tomatoes, parts of them, their plant cells or seeds, containing one or more of the DNA sequences according to Claim 1.

10. Tomatoes, parts of them, their plant cells or seeds, containing one or more of the RNA sequences according to one of Claims 2 or 3.

11. Use of ribozymes to inhibit synthesis of maturation enzymes in plants.

12. Use according to Claim 11, characterized in that the plants are fruit-bearing plants.

13. Use according to Claim 12, characterized in that the fruit-bearing plants are tomatoes.

14. Use according to one or more of Claims 11 to 13, characterized in that the RNA sequence according to Claims 2 or 3 is used as the ribozyme.

Patent claims for the following contracting state: ES

1. Process for producing a ribozyme-coding gene or gene fragment with the DNA sequence

1. Process for producing a ribozyme-coding gene or gene fragment with the DNA sequence

a) [See original for sequences.]

b)

c)

by synthesis of oligonucleotides, characterized in that oligonucleotides each having initial and final sequences of 5, preferably 7 to 10 nucleotides, are synthesized which, considered as a whole, are complementary to a DNA sequence of the maturation enzyme to be inhibited and which are separated by the intervening DNA sequence, consisting in part of specific nucleotides required for the functionality of the ribozyme and in part of variable nucleotides.

2. Process for producing RNA with ribozyme activity from the sequence

[See original for sequence.]

Ribozyme

Loop

characterized in that an oligonucleotide of the sequence

[See original for sequence.]

is synthesized, in which
K represents nucleotides A, C, G or T complementary to plant maturation enzyme RNA,

V represents variable nucleotides A, C, G or T,
V_L represents variable nucleotides A, C, G or T, whereby the nucleotide number of V_L is a number from 0 to 550 and

K', V', V_L' are each nucleotides A, C, G or T complementary to K, V, and V[L],

and is cloned in an intermediate vector with a plant promoter, then cloned together with the plant promoter in a binary plant vector and is transformed with the plasmid DNA of a plant so obtained.

3. Process according to Claim 2, characterized in that an RNA of the sequence

a) [See original for sequence.]

b) [See original for sequences.]

or

c)

is synthesized.

4. Use of ribozymes to inhibit synthesis of maturation enzymes in plants.

5. Use according to Claim 4, characterized in that the plants are fruit-bearing plants.

6. Use according to Claim 5, characterized in that the fruit-bearing plants are tomatoes.

7. Use according to one or more of Claims 4 to 6, characterized in that the RNA sequence according to Claims 2 or 3 is used as the ribozyme.